

Optimized protocol for the detection of multifunctional epitope-specific CD4+ T cells combining MHC-II tetramer and intracellular cytokine staining technologies

This is the peer reviewed version of the following article:

Original:

Pastore, G., Carraro, M., Pettini, E., Nolfi, E., Medaglini, D., Ciabattini, A. (2019). Optimized protocol for the detection of multifunctional epitope-specific CD4+ T cells combining MHC-II tetramer and intracellular cytokine staining technologies. FRONTIERS IN IMMUNOLOGY, 10, 1-10 [10.3389/fimmu.2019.02304].

Availability:

This version is available <http://hdl.handle.net/11365/1079892> since 2019-09-23T12:12:42Z

Published:

DOI:10.3389/fimmu.2019.02304

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1 **Optimized protocol for the detection of multifunctional epitope-specific CD4⁺ T cells combining**
2 **MHC-II tetramer and intracellular cytokine staining technologies**

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16
17 **Running title:** Multifunctional tetramer-specific CD4⁺ T cells

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19 **Keywords:** MHC-II tetramers, ICS, cytokines, multifunctional T cells, flow cytometry, immune
20 response, vaccination

21 **Abstract**

22

23 Analysis of multifunctional CD4⁺ T cells is fundamental for characterizing the immune responses to
24 vaccination or infections. Peptide-MHC tetrameric complexes represent a powerful technology to
25 detect antigen-specific T cells by the specific binding to their T cell receptor, and their combination
26 with functional assays is fundamental for characterizing the antigen-specific immune response. Here
27 we optimized a protocol for the detection of multiple intracellular cytokines within epitope-specific
28 CD4⁺ T cells identified by the MCH class II tetramer technology. The optimal procedure for assessing
29 the functional activity of tetramer-binding CD4⁺ T is based on the simultaneous intracellular staining
30 with both MHC tetramers and cytokine-specific antibodies upon *in vitro* restimulation of cells with
31 the vaccine antigen. The protocol was selected among procedures that differently combined the steps
32 of cellular restimulation and tetramer staining with intracellular cytokine labelling.
33 This method can be applied to better understand the complex functional profile of CD4⁺ T cell
34 responses upon vaccination or infection.

1. Introduction

The study of the CD4⁺ T cell activation and effector function is fundamental in the characterization of immune responses to vaccination (1).

CD4⁺ T cells play a central role in mediating vaccine immune responses by shaping both the humoral and cellular immunity (2). Activated CD4 T cells are critically involved in providing cognate help to B cells for production of protective antibodies, and modulate the functions of macrophages and CD8⁺ cytotoxic T cells through cytokines secretion. The characterization of the cytokine production of antigen-specific T cells is therefore of critical importance to profile vaccine immune response. The direct and specific method for identifying antigen-specific CD4⁺ T cells is based on the major histocompatibility complex (MHC) tetramer staining technique (3). This procedure allows the identification of specific T cells due to the selective and multivalent binding of tetramer MHC–peptide complexes to the T cell receptors (TCR) (3,4) and has been used for characterizing the primary and recall antigen-specific CD4⁺ T cell responses in many pre-clinical and human studies (1,5–9).

The effector function of antigen-reactivated T cells is commonly measured by flow cytometry-based intracellular cytokine staining (ICS) that allows the simultaneous phenotypic characterization and cytokine detection within single cells (10,11). The characterization of intracellular cytokines allows to identify activated CD4⁺ T cells capable of producing more than one cytokine, and the analysis of these multifunctional/polyfunctional cells is important for characterizing the immune response elicited by the vaccination or natural infections (12). Polyfunctional CD4⁺ T cells secreting IFN- γ , TNF- α and IL-2 have been proposed as a major component of immune response that correlates with mouse protection against challenge with *Leishmania major* (13). In tuberculosis (TB), it has not been clarified if the frequency and quality of polyfunctional CD4⁺ T cell responses elicited in mice by different types of vaccines correlate with protective immunity (14–17), while human studies have shown that a consistent response of CD4⁺ T cells co-expressing IFN- γ , TNF- α and IL-2 was associated with acute TB infection (18). Tetramer labeling and intracellular cytokine staining are generally not recommended to be performed concurrently since the *in vitro* antigen restimulation can induce TCR internalization, thus losing the possibility of detecting epitope-specific CD4⁺ T cells using tetramers (19).

In order to identify a protocol for the detection of intracellular cytokine production within the activated epitope-specific CD4⁺ T cells, we assessed different strategies that combined cellular restimulation (with the vaccine antigen or tetramers) and tetramer staining (extracellular or intracellular) with intracellular cytokine labelling. The different procedures were tested in

splenocytes from mice immunized with the chimeric tuberculosis vaccine antigen H56 (20) mixed with the adjuvant CAF01 (21), a model vaccine formulation deeply characterized in preclinical studies for its capacity of inducing both humoral and cellular responses (9,22–24). H56 is a fusion protein of *M. tuberculosis* antigens Ag85B, ESAT-6, and Rv2660, and the H56-specific CD4⁺T cell response can be monitored by employing Ag85B_{280–294}-complexed MHC class II tetramers (8). The different procedures were tested also in another experimental setting, in which mice were immunized with the model chicken ovalbumin antigen, and the CD4⁺ T cell response was assessed employing tetramers specific for the epitope_{325–335} (25).

The comparative analysis of the different protocols has permitted to optimize the procedure for identifying the multifunctional profile of tetramer-specific CD4⁺ T cells performing intracellular staining with both tetramers and cytokine-specific antibodies, upon antigen restimulation. This method represents a helpful tool for identifying epitope-specific CD4⁺ T cells and analyzing their specific effector function.

2. Materials and methods

2.1 Mice

Female C57BL/6 mice, purchased from Charles River (Lecco, Italy) were housed under specific pathogen-free conditions in the animal facility of the Laboratory of Molecular Microbiology and Biotechnology (LA.M.M.B.), Department of Medical Biotechnologies at University of Siena, and treated according to national guidelines (Decreto Legislativo 26/2014). The protocol was approved by the Italian Ministry of Health (authorization n° 1004/2015-PR, 22 September 2015).

2.2 Immunizations

Groups of 10-12 mice were immunized by the subcutaneous route at the base of the tail with the chimeric tuberculosis vaccine antigen H56 (2 µg/mouse) combined with the adjuvant CAF01 (250µg dimethyldioctadecylammonium and 50µg trehalose dibehenate/mouse, both kindly provided by Statens Serum Institut, Denmark) and boosted with a lower dose of H56 alone (0.5 µg/mouse) 4 weeks later. Another experiment was performed immunizing mice with albumin from hen egg white (OVA, 25 µg/mouse, Sigma-Aldrich) combined with the adjuvant CAF01, and boosted with OVA alone. The formulations containing antigens and CAF01 were injected in a volume of 150µl/mouse of Tris 10 mM, while the formulations containing H56 and OVA alone in a volume of 100µl/mouse of 1X Dulbecco's Phosphate Buffered Saline (1X PBS). Mice were sacrificed 5 days after boosting.

2.3 Sample collection and cell preparation

Spleens collected from mice were mashed onto 70µm nylon screens (Sefar Italia, Italy) and washed in complete RPMI (cRPMI) medium [RPMI (Lonza, Belgium), 100 U/ml penicillin/streptomycin, and 10% fetal bovine serum (Gibco, USA)] for 10 min at 300 g at 4°C. Splenocytes were treated with red blood cell lysis buffer (1X, eBioscience, USA) for 4 min. Following centrifugation at 300 x g at 4°C for 10 min, cells were washed with 1X PBS and counted with cell counter (Bio-Rad, USA).

2.4 Protocols and reagents

Six different protocols for detecting intracellular cytokines within activated epitope-specific CD4⁺ T splenocytes that differently combined cellular restimulation, tetramer staining and cytokine labelling, were assessed (Figure 1). Protocols were assessed in two different experimental settings, in which mice were immunized with H56 or OVA antigens, and the CD4 T cell response was analysed employing two different tetramers, specific for the H56 or OVA epitopes, respectively.

116 *Protocol 1:* Splenocytes (2×10^6 /well) were cultured in a round-bottom 96-well plate with H56 protein
117 (2 μ g/ml) or OVA (50 μ g/ml), anti-CD28 and anti-CD49d costimuli (both 2 μ g/ml, eBioscience) at
118 37°C, 5%CO₂ for 6h, with Brefeldin A (BFA, 5 μ g/ml, Sigma-Aldrich) and monensin solution (1 \times ,
119 eBioscience) added during the last 5 h of incubation. Cells were washed with cRPMI for 7 min at 300
120 g at 4°C, labelled with Fixable Viability Stain 780 staining (FVS780, BD Biosciences, 1:1000, 100
121 μ l/well) for 20 min at RT in the dark, and washed twice in PBS. Cells were fixed and permeabilized
122 for 20 min at 4°C with BD Cytofix/Cytoperm (Becton Dickinson). Samples were blocked for 30 min
123 at 4°C in Fc-blocking solution (5 μ g/ml of CD16/CD32 mAb, eBioscience, USA) and stained for 1 h
124 at room temperature (RT) with PE-conjugated I-A (b) *M. tuberculosis* Ag85B precursor 280-294
125 (FQDAYNAAGGHNAVF) tetramer (diluted 1:80, hereafter Tet-Ag85B) or with PE-conjugated I-A
126 (b) chicken ova 325-335 (QAVHAAHAEIN) tetramer (diluted 1:50, hereafter Tet-OVA; both
127 tetramers were kindly provided by NIH MHC Tetramer Core Facility, Emory University, Atlanta,
128 GA, USA) diluted in Perm/wash buffer. In the last 20 minutes of tetramer incubation, the following
129 mix of fluorescent antibodies was added: APC-conjugated anti-CD3 (clone 145-2C11), BB700-
130 conjugated anti-CD4 (clone RM-5), APC-R700-conjugated, anti-CD44 (clone IM-7), BV786-
131 conjugated anti-IFN- γ (clone XMG1.2), BV650-conjugated anti-TNF- α (clone MP6-XT22), BV421-
132 conjugated anti-IL-17A (clone TC11-18H10), PE-CF594-conjugated anti-IL-2 (clone JES6-5H4),
133 (all antibodies were purchased from BD Biosciences). All antibodies and tetramer were titrated for
134 optimal dilution.

135 *Protocol 2:* Splenocytes were cultured with the respective antigens and costimuli as in protocol 1,
136 then were washed and stained with the respective tetramers for 1h at RT. Cells were labelled with
137 FVS780, fixed and permeabilized with BD Cytofix/Cytoperm and stained with the mix of fluorescent
138 antibodies for 20 minutes at RT.

139 *Protocol 3:* Splenocytes were stained with the respective tetramers for 1h at RT, washed and
140 stimulated with the respective antigens and anti-CD28 and anti-CD49d costimuli at 37°C for 6h, with
141 Brefeldin A and monensin solution added during the last 5 h of incubation. Cells were labelled with
142 FVS780, fixed and permeabilized with BD Cytofix/Cytoperm and stained with the mix of fluorescent
143 antibodies for 20 minutes at RT.

144 *Protocol 4:* Splenocytes were cultured with the respective tetramers and costimuli for 1h at RT,
145 washed and added with BFA and monensin solution at 37°C for 5 h. Cells were labelled with FVS780,
146 fixed and permeabilized with BD Cytofix/Cytoperm and stained with the mix of fluorescent
147 antibodies for 20 minutes at RT.

148 *Protocol 5:* Splenocytes were cultured with the respective tetramers and costimuli for 1h at RT and
149 for 5h at 37°C in the presence of BFA and monensin. Cells were labelled with FVS780, fixed and

150 permeabilized with BD Cytofix/Cytoperm and stained with the mix of fluorescent antibodies for 20
151 minutes at RT.

152 *Protocol 6:* Splenocytes were cultured with the respective tetramers and costimuli for 6h at 37°C,
153 with BFA and monensin during the last 4h of incubation. Cells were labelled with FVS780, fixed and
154 permeabilized with BD Cytofix/Cytoperm and stained with the mix of fluorescent antibodies for 20
155 minutes at RT.

156

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159 **2.5 Flow cytometry**

160 About 7×10^5 stained cells from each protocol were acquired on BD™ LSRFortessa X20 flow
161 cytometer (BD Biosciences) and stored. Data analysis was performed using FlowJo v10 (TreeStar,
162 USA), and the evaluation of different cytokines co-expression was performed using the FlowJo
163 Boolean gate platform. Fluorescence minus one (FMO) controls were performed for all fluorescence
164 and used for gating setting.

165

166

167 **2.6 Statistical analysis**

168 Kruskal-Wallis test, followed by Dunn's post test for multiple comparisons, was used to assess the
169 statistical difference between protocols. A P value ≤ 0.05 was considered significant. Analysis were
170 performed using GraphPad Prism v7 (GraphPad Software, USA).

3. Results

In order to optimize the protocol for the detection of intracellular cytokines within activated epitope-specific CD4⁺ T cells, we tested different procedures in splenocytes from mice parenterally immunized with two different antigens, the chimeric TB vaccine antigen H56 or OVA, combined with the liposome adjuvant CAF01, 5 days after the booster immunization. The induction of Ag-specific CD4⁺ T cells producing cytokines was assessed combining antigen restimulation and tetramer staining, followed by intracellular cytokine detection (Figure 1). In protocols 1-3 splenocytes were restimulated with the respective antigens, added before (protocols 1 and 2) or after (protocol 3) tetramer staining. In protocols 4-6 the restimulation step was performed directly with epitope-complexed MHC II tetramers, that were therefore used not only as staining tool, but also as functional stimulus. The comparison of results obtained following the different strategies, and tested with two different antigens, has permitted to optimize the procedure for identifying the cytokine profile of tetramer-specific CD4⁺ T cells.

3.1 Identification of tetramer-specific CD4⁺ T cells producing cytokines

H56-specific CD4⁺ T cells were identified using the Ag85B₂₈₀₋₂₉₄-complexed MHC class II tetramers specific for the immunodominant epitope of Ag85B (26), which is part of the chimeric H56 protein, while OVA-specific CD4⁺ T cells using the chicken OVA₃₂₅₋₃₃₅-complexed MHC class II tetramers. Tetramer positive cells (Tet-Ag85B⁺ or Tet-OVA⁺) were identified as live single CD3⁺ CD4⁺ CD44⁺ cells, and TNF- α , IFN- γ , IL-17 and IL-2 cytokines were detected within gated Tet-Ag85B⁺ cells. All gates were defined on the bases of the respective FMO controls. Staining specificity was determined using a control tetramer complexed with an unrelated antigen that showed a level of staining below 0.02% (data not shown). The identification of Tet-Ag85B⁺ cells in the different six protocols (Figure 2 A), and their intracellular cytokine production (Figure 2 B) are shown.

ICS protocol typically includes an antigen stimulation step, that is crucial for activation of effector function of CD4⁺ T cells. Nevertheless, this step induces the internalization of TCR molecules, thus negatively impacting on the tetramer staining procedure. To overcome this limitation, we assessed a strategy based on the antigen stimulation phase followed by permeabilization and fixation of cells and subsequent tetramer staining (Figure 1, protocol #1). Using this procedure, that allows to identify both extra and intracellular TCR molecules, we detected 0.53% of Tet-Ag85B⁺ cells (Figure 3A, orange box). This frequency was significantly higher compared to protocol 2, in which splenocytes were firstly stimulated with H56 antigen and then labelled with the specific tetramer (Figure 1,

204 protocol #2) allowing an identification of only a 0.2 % of tetramer-positive T cells (Figure 3A, light
205 green).

206 The impact of the tetramer staining performed before antigen restimulation was also evaluated (Figure
207 1, #3). This procedure allowed to detect a frequency of 0.37% of Tet-Ag85B⁺ T cells that was higher
208 compared to protocol 2 while was lower respect to protocol 1(Figure 3A dark green box). The higher
209 number of Tet-Ag85B⁺ T cells detected in protocol 1 could be due to the effect of prior antigen
210 restimulation that is known to induce the formation of large clusters of TCR molecules thus increasing
211 tetramer binding avidity (27).

212 In protocols 4, 5 and 6, there was no antigen stimulation and the Ag85B₂₈₀₋₂₉₄-complexed MHC class
213 II tetramers were used not only for identifying but also for stimulating antigen-specific CD4⁺ T cells
214 (Figure 1, protocols #4, 5 and 6). As expected, the frequencies of Tet-Ag85B⁺ T cells detected in
215 protocol 4 were comparable to those of protocol 3 (Figure 3A, light blue and dark green box). In
216 protocols 5 and 6, in which a tetramer incubation phase of 6 hours was performed, frequencies of
217 1.07 and 1% of Tet-Ag85B⁺ T cells were observed, significantly higher respect to H56-stimulated
218 samples (Figure 3A, $P \leq 0.05$ and $P \leq 0.001$ compared to protocols 3 and 2, respectively). A similar
219 behavior was observed in mice immunized with the OVA antigen, in which the OVA-specific CD4⁺
220 T cells were identified using the OVA₃₂₅₋₃₃₅ peptide-complexed tetramer (Figure 3B). This shows that
221 tetramers with different peptide specificity, respond in a very similar way to the *in vitro* staining
222 procedures assessed in the different protocols.

223 The effector function of both Tet-Ag85B⁺ and Tet-OVA⁺ T cells identified with the different
224 strategies, was analyzed by measuring the intracellular production of 4 different cytokines using
225 multiparametric flow cytometry. As shown in figure 3 C and D, the highest percentage of cells
226 producing intracellular cytokines was detected for both tetramers in protocols 1, 2 and for Tet-
227 Ag85B⁺ also in protocol 3, compared to protocols 4, 5 and 6. These data show that in absence of
228 antigen stimulation, despite the high frequencies of tetramer-binding CD4⁺ T cells (protocols 5 and
229 6, Figure 3 A and B), a significant lower cytokines production is induced (Figure 3 C and D)
230 highlighting the importance of the *in vitro* re-stimulation step with the vaccine antigen to effectively
231 stimulate the effector function of antigen-specific CD4⁺ T cells.

232

233 **3.2 Evaluation of tetramer-specific CD4⁺ T cells multifunctional profile.**

234 In order to have a picture of the multifunctional profiles of T cells elicited by immunization and
235 detected by the different experimental procedures, a Boolean analysis of data was performed within
236 Tet-Ag85B⁺ cells (Figure 4A). A significant amount of cells positive for all the four cytokines or for
237 TNF- α , IFN- γ , and IL-2 were observed in protocols 1 and 3 compared to protocols 4 and 5 ($P \leq 0.05$).

238 Cells producing only IFN- γ were instead significantly higher in protocols 5 and 6 compared to
239 protocol 2 ($P \leq 0.01$). The analysis of the frequency of cells producing two or more cytokines
240 (multifunctional), a single one (single) or no cytokines in each protocol shows that the frequency of
241 multifunctional Tet-Ag85B⁺ T cells was higher in protocols 1, 2 and 3 (79, 68, 69 % respectively)
242 that included the antigen restimulation. Lower frequencies of multifunctional cells were observed in
243 protocols 4, 5 and 6 (12, 7, 18 % respectively) in which most of cells did not produce any cytokine
244 (75, 74, 68 %) (Figure 4B). The intracellular staining with the tetramer performed in protocol 1,
245 allowed to detect the highest percentage of multifunctionality, while no differences were observed
246 among protocols 2 and 3. Therefore the optimal strategy of staining that allows to identify
247 multifunctional T-helper cells among tetramer-specific CD4⁺ T cells was the protocol #1.

248
249 In conclusion, our comparative analysis, confirmed for two different antigens and their respective
250 tetramers, has shown that the optimal strategy for identifying the multifunctional cytokine profile of
251 tetramer-specific CD4⁺ T cells is the procedure #1, in which the antigen restimulation phase is
252 followed by the intracellular tetramer staining. Indeed, this protocol allows to detect a significant
253 amount of tetramer-specific T cells, their multifunctional activity, and it allows to reduce the staining
254 time, by adding cytokine-specific antibodies in the last 20 minutes of tetramer incubation. A detailed
255 description of protocol #1 is reported in Figure 5.

Discussion

In this study we optimized a flow cytometric protocol for identifying at the single-cell level multifunctional epitope-specific CD4⁺ T cells, elicited by immunization. Demonstrating pros and cons of different protocols, we showed that the optimal procedure for the simultaneous detection of epitope-specific CD4⁺ T cells and their effector function is based on the antigenic stimulation of cells combined with a single step of cytokine and tetramer staining in permeabilized cells (Figure 5). Our analysis was based on the comparison of different experimental procedures, tested with two different epitope-specific tetramers, in which the steps of antigen restimulation, tetramer and cytokine staining were differently combined. The systematic analysis of different procedures performed in the same samples has offered the possibility of selecting the optimal protocol among different strategies. The results have been confirmed with tetramers specific for two different antigens, thus strengthening the possible application of the selected procedure to the characterization of the complex functional profile of CD4⁺ T cell responses upon vaccination or infection.

Most of the studies of intracellular cytokine production within tetramer positive cells, have been conducted in CD8⁺ T cells (28–30), while few works have been performed in CD4⁺ T cells with MHC class II tetramers (31–33) and none has compared different protocols in a systematic way. Even though a direct comparison with the present study is difficult due to different experimental settings, i.e the use of T cell clones or human CD4⁺ T cells, prolonged incubation with antigen for cell activation, magnetic bead enrichment of tetramer positive cells before ICS staining, we can generally observe that the tetramer staining was performed extracellularly, often before the antigen stimulation step. Our analysis clearly demonstrates that antigenic stimulation is necessary for an efficient reactivation of the cellular effector function, and the same stimulation effect can not be obtained with the direct incubation of cells with epitope-complexed MHC tetramers, also when prolonged for 6 hours (protocols 5-6). Nevertheless, many studies have demonstrated that ligation of TCR by processed antigen induces TCR internalization and a subsequent down-modulation of its cell surface expression (19,34). Indeed, in protocol 2, in which antigen stimulation was performed before the extracellular tetramer staining the frequency of tetramer positive cells was significantly lower compared to protocol 1.

Here, using tetramers specific for two different antigens, we have shown the efficiency of tetramer staining performed in permeabilized cells (protocol 1) that allows to detect both surface expressed and internalized TCR molecules resulting in the identification of the highest percentages of tetramer-binding cells. This procedure stained epitope-specific CD4⁺ T better than protocol 3 in which labeling with tetramer was performed before antigen stimulation. This can be due to the lower avidity of

290 tetramer binding to TCR molecules in the absence of cell activation by antigen stimulation. Indeed,
291 cellular activation is known to induce the TCR reorganization with the generation of large clusters
292 of TCR molecules (27) that increase the strength of tetramer binding.

293 Analysis of multifunctional CD4⁺ T cells is of critical importance for in depth characterization of
294 immune responses to vaccination both in pre-clinical and clinical studies. It is therefore essential to
295 have a protocol that optimally combines the identification of antigen-specific T cells with the analysis
296 of their cytokine profile. Here, we show the possibility to combine, upon antigen stimulation, tetramer
297 and intracellular cytokine staining in permeabilized cells allowing the identification of an higher
298 number of polyfunctional tetramer positive CD4 T cells. The amount of cells producing all the four
299 cytokines, or coexpressing two or three cytokines (especially TNF- α , IFN- γ , and IL-2) was indeed
300 higher compared to the other protocols tested. Significant lower levels of multifunctional cells were
301 observed when tetramers were used both as stimulus and as staining (protocols 4, 5 and 6). Indeed,
302 even though a higher percentage of tetramer-binding T cells was identified by protocols 5 and 6, about
303 70% were negative for cytokine production, respect to 14% observed in protocol 1, demonstrating
304 that the binding of epitope-complexed MHC class II molecules to TCR in the presence of CD49d and
305 CD28 costimuli is not sufficient for effectively reactivating multifunctional antigen-specific CD4⁺ T
306 cells.

307 The functional characterization of CD4⁺ T cells described by the protocols analyzed here, is
308 particularly suitable for pre-clinical studies, in which sufficient quantity of CD4⁺ T cells can be easily
309 identified in draining lymphoid organs such as lymph nodes or spleens, while in humans it is generally
310 more complicated because the frequency of antigen-specific CD4⁺ T cells in blood are low and often
311 undetectable (6). Moreover, the use of MHC tetramers requires prior knowledge of the peptide epitope
312 and host MHC haplotype, a limitation that can be easily circumvented in inbred animals.

313 In conclusion, in the present work we have selected an optimized protocol for identifying epitope-
314 specific CD4⁺ T cells and their effector function, combining antigenic stimulation of cells with the
315 intracellular staining of TCR molecules and cytokines. Antigenic restimulation, performed at the
316 beginning of the procedure, allows the activation of cells and elicits multiple cytokine production,
317 but at the same time it promotes the down regulation of surface TCR expression that is resolved by
318 the intracellular tetramer staining. This procedure allows also to reduce the total protocol time, since
319 tetramer, surface marker and cytokine staining are combined in a single staining step.

320 This protocol allows to better understand the complex functional profile of T cell responses upon
321 vaccination or natural infection, and it can be instrumental for the dissecting the immune response to
322 vaccination.

323 **Acknowledgments**

324 The authors acknowledge the NIH Tetramer Core Facility (contract HHSN272201300006C) for
325 provision of MHC class II tetramers, and Staten Serum Institute for provision of H56 and CAF01
326 reagents.

327 This study has been carried out with financial support from the Commission of the European
328 Communities, Seventh Framework Programme, contract HEALTH-2011-280873 “Advanced
329 Immunization Technologies” (ADITEC), and Horizon 2020 Framework Programme, grant number
330 730964 (TRANSVAC).

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334 **Author Contributions**

335 GP, DM, AC conceived and designed the experiments; GP, MC, EP: performed the experiments; GP,
336 MC, AC, EP, EN: analyzed the data; GP, AC: wrote the paper; DM, AC: critically revised the
337 manuscript. All authors read and approved the final manuscript.

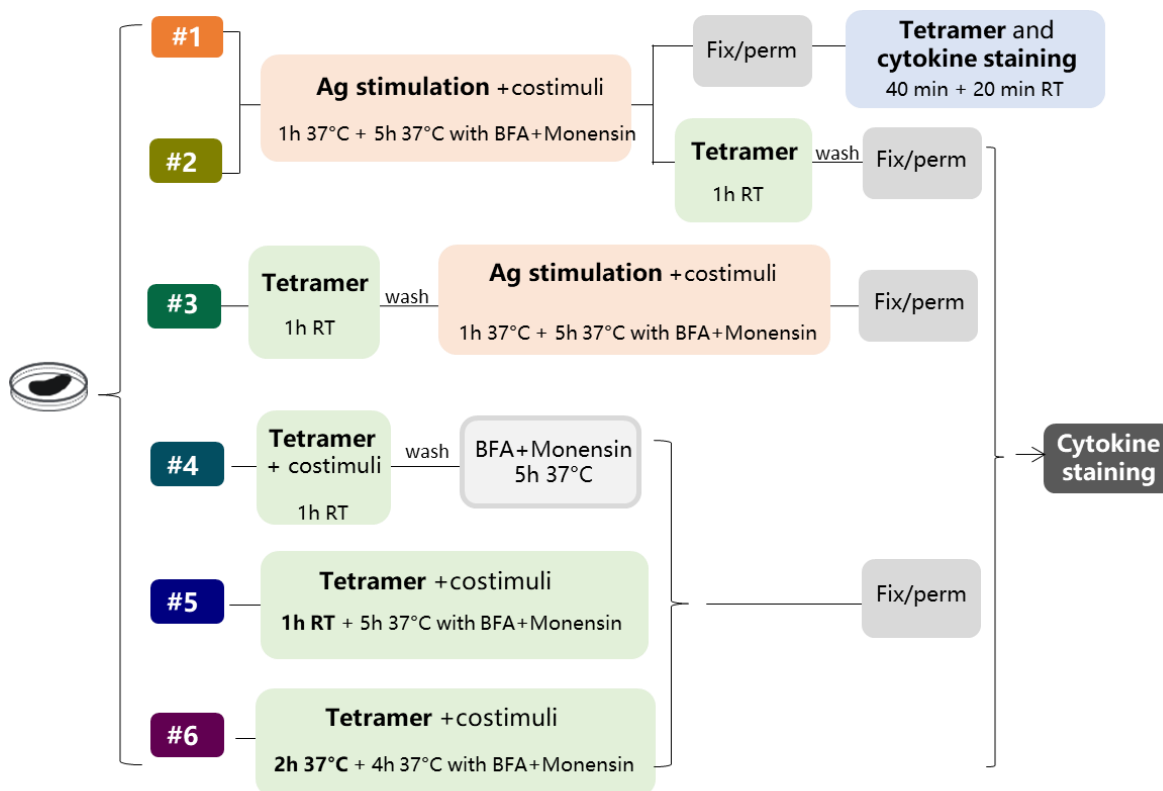
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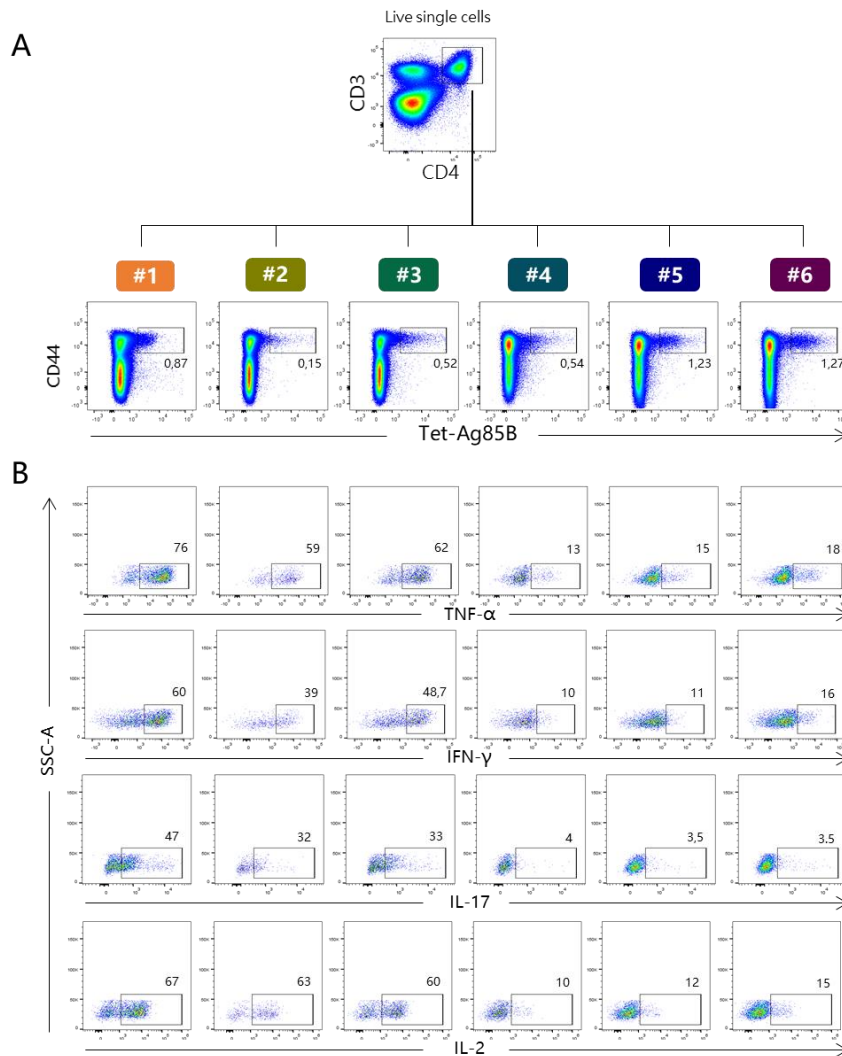
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437 **Figure 1. Study design.** Six different protocols, combining antigen stimulation and tetramer staining with
 438 intracellular cytokine labelling, were used for detecting antigen-specific CD4+ T cell producing cytokines in
 439 splenocytes of mice immunized with two different antigens, H56 or OVA, and CAF01 adjuvant, 5 days after
 440 booster immunization. In protocols 1-3 splenocytes were restimulated with the respective antigen (Ag, pink
 441 box), added before (protocols 1 and 2) or after (protocol 3) tetramer staining (green box), while in protocols
 442 4-6 the restimulation step was performed directly with Ag85B or OVA epitope-complexed MHC II tetramers.
 443 Anti-CD28 and anti-CD49d (co-stimuli) were added with Ag (protocol 1-3) or with tetramers (protocols 4-6).
 444 After Ag or tetramer incubation, cells were treated with Brefeldin A and Monensin for 4-5 h at 37°C, fixated
 445 and permeabilized (gray box) and finally stained with anti-cytokines antibodies (dark gray box), except for
 446 protocol 1, in which tetramer staining was performed in fixed and permeabilized cells together with cytokine
 447 staining. In protocol 6, tetramer staining was performed for 2 h at 37°C.



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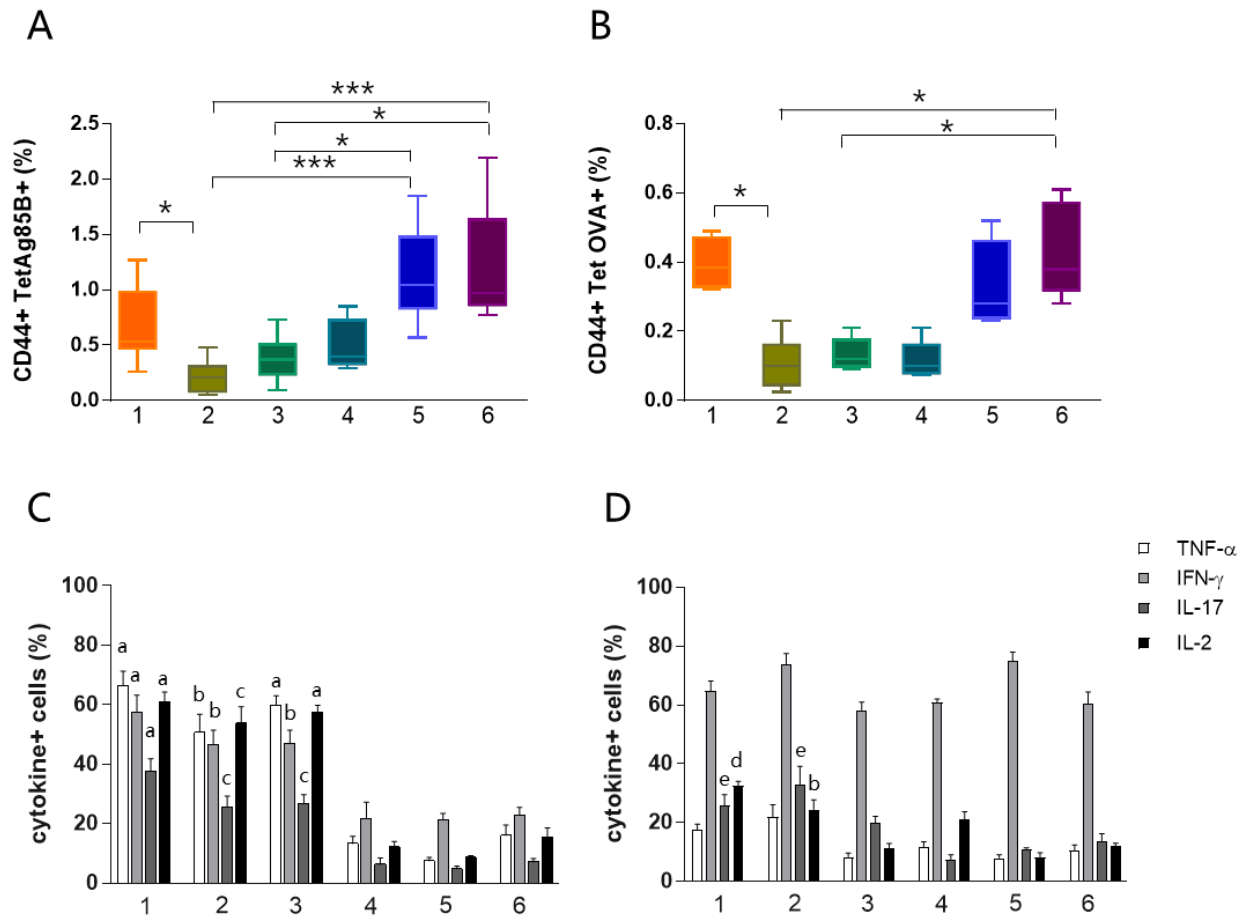
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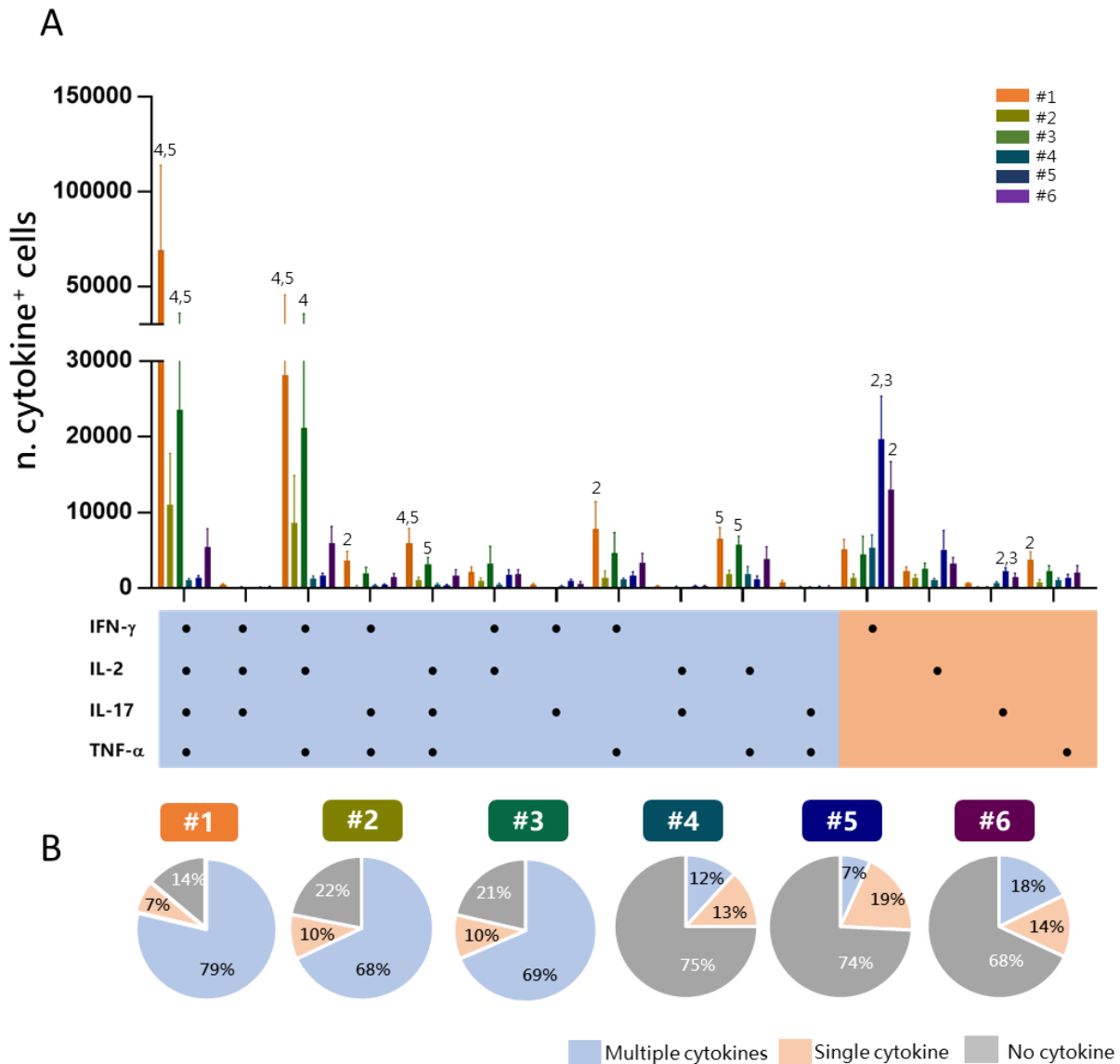
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Figure 2. Flow cytometric analysis of Tet-Ag85B+ T cells producing cytokines. A. Ag85B-tetramer binding T cells were identified among live single CD3+ CD4+, as CD44^{high} Tet-Ag85B+ cells in the six different protocols, and the frequencies of positive cells are reported within the dot plots. B. Intracellular production of TNF α , IFN γ , IL-17 and IL-2 cytokines assessed within the Tet-Ag85B+ T cells in the six different protocols. Frequencies of positive cells are reported within the dot plots. Gates were defined on the respective FMO controls.



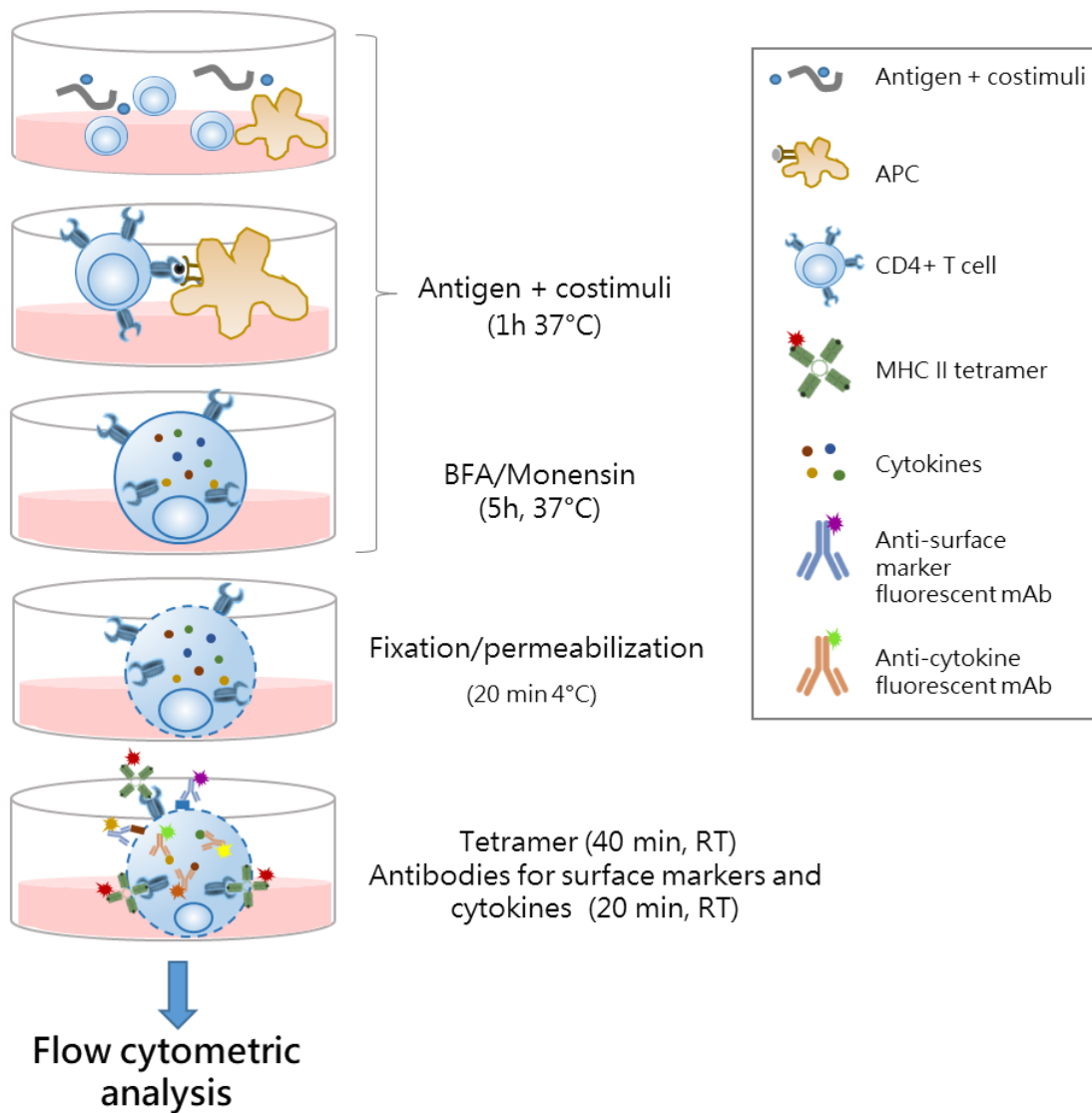
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456 **Figure 3. Identification of tetramer-specific CD4+ T cells and their cytokine production.** Tetramer-
 457 specific CD4+ T cells and their cytokine production were assessed in splenocytes treated with the different
 458 protocols reported in Figure 1. A-B. Box plots of the frequencies of Tet-Ag85B+ (A) and Tet-OVA+ (B) T
 459 cells respect to CD4+ T cells, detected employing protocols 1-6, as reported in x axis. Values are reported as
 460 mean \pm SEM of 10-12 mice, obtained in three independent experiments. Kruskal-Wallis test, followed by
 461 Dunn's post test for multiple comparisons, was used to assess the statistical difference between protocols (* P
 462 ≤ 0.05 , ** $P \leq 0.01$, *** $P \leq 0.001$). C-D. Frequencies of TNF- α , IFN γ , IL-17 and IL-2 positive cells among
 463 Tet-Ag85B+ (C) and Tet-OVA+ (D) cells, employing protocols 1-6, as reported in x axis. Values are reported
 464 as mean \pm SEM of 10-12 mice, obtained in three independent experiments. The significant difference between
 465 each cytokine among the different protocols, according to the Kruskal-Wallis test followed by Dunn's post test
 466 for multiple comparisons ($P \leq 0.05$), is reported with letters above the error bars; "a", significant difference
 467 versus protocols 4, 5 and 6; "b" versus protocol 5; "c" versus protocols 4 and 5; "d" versus protocols 3, 5 and
 468 6; "e" versus protocol 4.



469

470 **Figure 4. Multifunctional response of Tet-Ag85B⁺ T cells.** Multifunctional profiles of Tet-Ag85B⁺ T cells
 471 detected by the different experimental procedures. A. Histograms represent the number of Tet-Ag85B⁺ T cells
 472 producing different combinations of cytokines shown on the x axis, detected employing the different protocols.
 473 Responses are grouped and color coded according to the functionality (orange for single cytokine, light blu for
 474 two or more cytokines). Values are reported as mean \pm SEM of 10-12 mice, obtained in three independent
 475 experiments, and the numbers above the error bars indicate which protocols are significantly different
 476 according to the Kruskal-Wallis test, followed by Dunn's post test for multiple comparisons ($P \leq 0.05$). B. Pie
 477 charts of the 6 protocols, in which each slice of the pie represents the fraction of Tet-Ag85B⁺ T cells producing
 478 two or more cytokines (multiple cytokines, light blue), a single one (orange) or none (grey). Frequencies are
 479 reported within each slice.



480

481 **Figure 5. Optimal procedure for identifying multifunctional tetramer-specific CD4⁺ T cells.** Schematic
 482 overview of the protocol optimized for the detection of multifunctional epitope-specific CD4⁺ T cells.
 483 Splenocytes are cultured in 96-well plates with antigen and costimuli for 1h at 37°C in order to allow antigen
 484 presentation by APC to cognate epitope-specific CD4⁺ T cells. Antigen stimulation elicits reactivation of
 485 effector function of CD4⁺ cells, and TCR internalization. Brefeldin A (BFA) and monensin solution are added
 486 for the last 5 h of incubation to block cytokines secretion. Cells are fixed and permeabilized for 20 min at 4°C,
 487 and then simultaneously stained with MHC II tetramers (1 h, at RT) and surface markers/cytokine-specific
 488 antibodies (last 20 minutes). This allows to detect both surface expressed and internalized TCR molecules,
 489 with intracellular cytokines. The single-step staining procedure allow to reduce the complex time of the
 490 protocol. Stained samples are then analysed by flow cytometry.